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Activation of specific MEK-ERK cascade is necessary for TGFβ signaling and crosstalk with PKA and PKC pathways in cultured rat articular chondrocytes

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Summary

Objective: TGFβ is a potent stimulator of cell growth in cultured rat articular chondrocytes (CRAC). The stimulatory effect is mediated through the immediate induction of c-fos gene by activating ERK of MAPK. The present study was undertaken to investigate the upstream regulators involved in TGFβ-induced ERK activation in CRAC and to compare the results with the events in HepG2 cells.

Results: In vitro kinase and trans-reporting assays showed that TGFβ preferentially activated ERK and JNK pathways in CRAC and HepG2, respectively. ERK activation in CRAC was selectively inhibited by PD98059, a MEK inhibitor. Overexpression of wild or active forms of MEKK1, the upstream activator of ERK and JNK, decreased the TGFβ-induced 3TP-luciferase activity in CRAC. In contrast, in HepG2 dominant negative form of MEKK1 or SEK1 ligand-dependent reporter activity was diminished. Transfection of TAK1, another MAPKKK, also positively and negatively regulated 3TP transcriptional activity of HepG2 and CRAC, respectively. Activation of PKA by 8-bromo-cyclic AMP or forskolin, and inhibition of PKC by calphostin C, resulted in a significant decrease in 3TP activity as well as *in vitro* ERK kinase activity in CRAC.

Conclusions: The results indicate that TGFβ transduces a predominant signal pathway through MEK-ERK-Elk1, independent of MEKK1 or TAK1 pathway in CRAC. However, in HepG2, activation of MEKK1 and TAK1 is essential for TGFβ-induced signal transmission. The results also demonstrated that in CRAC, MEK-ERK pathway activated by TGFβ is negatively regulated by PKA cascade but transactivated by PKC.

Key words: TGFβ, MAPK, Articular chondrocytes.

Introduction

Transforming growth factor-β (TGFβ) superfamily, composed of TGFβ, bone morphogenic protein (BMP), activin and cartilage-derived growth factor (CDGF) subfamilies, regulates a variety of cellular processes including embryonic differentiation, extracellular matrix formation, cell proliferation and apoptosis.[1–5] In articular cartilage, TGFβ plays a critical role in cartilage development, metabolism and repair process following cartilage damage. TGFβ also stimulates chondrocyte differentiation by accumulating chondrocyte-specific gene expression such as type II collagen, aggrecan and link protein.[6,7] In addition, TGFβ can potentially inhibit the release of many catalytic factors, which are elevated in osteoarthrosis.[8,9] Based on the available evidence obtained from various *in vitro* and *in vivo* studies, TGFβ is considered a potentially useful agent for the treatment of arthritic conditions.[10,11]

TGFβ binds with a heteromeric complex of the heterogeneous type I and type II receptors and transmits signals through mitogen-activated protein kinase (MAPK) and Smads pathways.[5,12–14] In general, TGFβ acts as an inhibitor of cell growth in many cell types, especially epithelial and endothelial cells. Paradoxically, TGFβ is also a potent stimulator of proliferation of cultured rat articular chondrocytes (CRAC).[15] We have demonstrated recently that TGFβ directly stimulates the growth of chondrocytes by inducing c-fos.[16] We have also shown that TGFβ specifically transactivates extracellular regulated kinase (ERK) but not c-Jun N-terminal kinase (JNK) or p38MAPK of MAPK cascades, and that ERK activation is required for TGFβ-induced proliferation of CRAC.[17] These results suggest that specific activation of ERK is a key event leading to growth stimulation of CRAC. However, the exact MEK kinase 1 (MEKK1) that regulates MAPK/ERK kinase (MEK)–ERK pathway is still not clear at this stage. Furthermore, the mechanisms by which other regulatory molecules regulate ERK activation remain to be elucidated.

In this study, we investigated the possible signal transduction pathways responsible for activation of an ERK cascade following TGFβ stimulation in CRAC. For this, we examined the role of several upstream regulators that could modify MAPK cascades in CRAC and HepG2, which was one of hepatoma cell lines and has often been used for TGFβ signaling. Our results demonstrated that TGFβ transduces intracellular signals preferentially through different MAPK cascades between CRAC and HepG2; activation of the MEK–ERK pathway is essential for TGFβ signaling, but MEKK1 and TGFβ-activated kinase (TAK1) negatively

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regulates it in CRAC. In contrast, MEKK1 and TAK1 activation is required for signaling in HepG2. We also showed the presence of cross-talk pathways from protein kinase A (PKA) and protein kinase C (PKC) cascades for ERK activation in CRAC.

Materials and methods

MATERIALS

Five-week-old male Sprague–Dawley (SD) rats were purchased from Charles River Laboratories (Shizuoka, Japan). Lipofectin reagent, fetal bovine serum (FBS), DMEM and RPMI medium were obtained from Gibco Oriental (Tokyo, Japan). Recombinant human TGFβ1 was from AUSTRAL Biologicals (San Ramon, CA). The cell culture plates were from Becton Dickinson (Rutherford, NJ), Falcon (Lincoln Park, NY), Corning (Corning, NY) and Costar (Cambridge, MA). Luciferase assay system and β-galactosidase enzyme assay were purchased from Promega (Tokyo, Japan). PathDetect Elk1 or cJun trans-reporting system and N-terminal deleted constitutive active MEKK1 (MEKK CA) were from STRATAGENE (La Jolla, CA). PhosphoPlus p44/42 MAPK(Tyr204) antibody kit, phosphospecific cJun antibody kit and specific MEK1 inhibitor (PD98059) were purchased from New England Biolabs (Beverly, MA). All other reagents were obtained from Sigma Chemical (St Louis, MO).

CELL CULTURES

Chondrocytes were isolated from rat articular cartilage as described previously.[15] Isolated chondrocytes were cultured in DMEM which contained 10% FBS, and passaged once for the experiments. HepG2 cells were cultured with RPMI containing 5% FBS.

IN VITRO KINASE ASSAY OF ERK AND JNK

ERK and JNK activities were estimated by *in vitro* kinase assay as described previously by our laboratory.[17] Kinase reaction was performed at 30°C for 30 min in the presence of cold ATP together with Elk1 or cJun, which fused to GST protein. Samples were subjected to SDS-PAGE, blotted onto PVDF membranes, and immunoblotted with phospho-specific Elk1 (Ser383) or phospho-cJun (Ser63) antibody.

TRANS-REPORTER ASSAY OF ELK1 AND CJUN

Phosphorylation of Elk1 and cJun *in vivo* was quantified by trans-reporting system as described previously.[17] The assay is designed to detect endogenous JNK and/or ERK activity *in vivo*. The pFA-cJun or –Elk1 vector expresses a fusion protein of the functional domain of c-Jun or Elk1 following GAL4 DNA binding domain. The reporter vector, pFR-Luc contains a GAL4-response element upstream of the luciferase gene. Phosphorylated Elk1 or cJun forms a homodimer, leads to binding with GAL4 responsive element, resulting in the activation of the reporter gene.

P3TP LUCIFERASE ASSAY

Cultured rat articular chondrocytes and HepG2 with 80% and 50% confluence, respectively, were transiently transfected by Lipofectin. Twenty-four hours later, the cells were further incubated for 12 h with or without TGFβ. Cell extracts were then prepared and subjected to luciferase assay or β-galactosidase assay as recommended by the manufacturer.

Results

SPECIFIC ACTIVATION OF ERK AND JNK BY TGFβ IN CRAC AND HEPG2

We have previously demonstrated that ERK (but not JNK or p38 MAP) was activated by TGFβ in CRAC.[17] To examine whether this specific activation is a cell type specific phenomenon, we first compared the time-dependent activation of ERK and JNK in response to TGFβ by *in vitro* kinase assay in CRAC and HepG2. For this purpose, kinase reaction was performed with immunoprecipitated ERK or JNK and the specific substrate in the presence of ATP. The phosphorylated substrate was detected by immunoblotting with phospho-specific antibody. Similar to the previous experiments, treatment of CRAC with TGFβ caused a rapid activation of ERK at 5 min, which reached a peak level at 15 min, while no apparent activation of JNK was observed (Fig. 1A). In contrast, in HepG2, activation of ERK could not be detected. Instead, JNK was activated gradually with the peak level recorded 1 h after TGFβ stimulation. The specific activation of MAPK cascades by TGFβ was confirmed by the *in vivo* reporter assay. The expression vectors of GAL4-Elk1 or c-Jun and GAL4 DNA binding domain fused to the reporter gene were transiently transfected to cells, followed by measurement of luciferase activity. In CRAC, TGFβ stimulated the phosphorylation of Elk1, but not c-Jun (Fig. 1B). In contrast, phosphorylation of both Elk1 and c-Jun was stimulated by TGFβ in HepG2.

PD98059, AN INHIBITOR OF MEK PREFERENTIALLY SUPPRESSED TGFβ-INDUCED MEK–ERK PATHWAY IN CRAC

Since Elk1 could be a substrate of both JNK and ERK, we used PD98059, an inhibitor of MEK that selectively activates ERK in order to determine whether Elk1 phosphorylation observed in HepG2 was meditated through ERK or JNK. For this, we first determined the optimal concentrations of PD98059 for the inhibition of MEK activity in CRAC. Incubation of PD98059 resulted in a dose-dependent inhibition of *in vitro* kinase activity, in which 50 μM concentration completely abolished TGFβ induced ERK activation (Fig. 2A). Incubation of HepG2 with PD98059, however, did not influence Elk-1 phosphorylation, while the phosphorylation of Elk1 was significantly blocked in CRAC (Fig. 2B). Furthermore, PD98059 inhibited 3TP-lux luciferase activity of CRAC but not of HepG2 (Fig. 2C). Considered together, these results indicate that TGFβ transmits the signal though MEK-ERK-Elk1 pathway in CRAC, while in HepG2, TGFβ transmits the signal through JNK- mediated Elk1 and cJun pathways.

DIVERGENT ACTION OF MEKK AND SEK ON 3TP-LUX ACTIVITY BETWEEN CRAC AND HEPG2

To identify upstream transducers involved in TGFβ-induced activation of ERK or JNK, several forms of MEKK

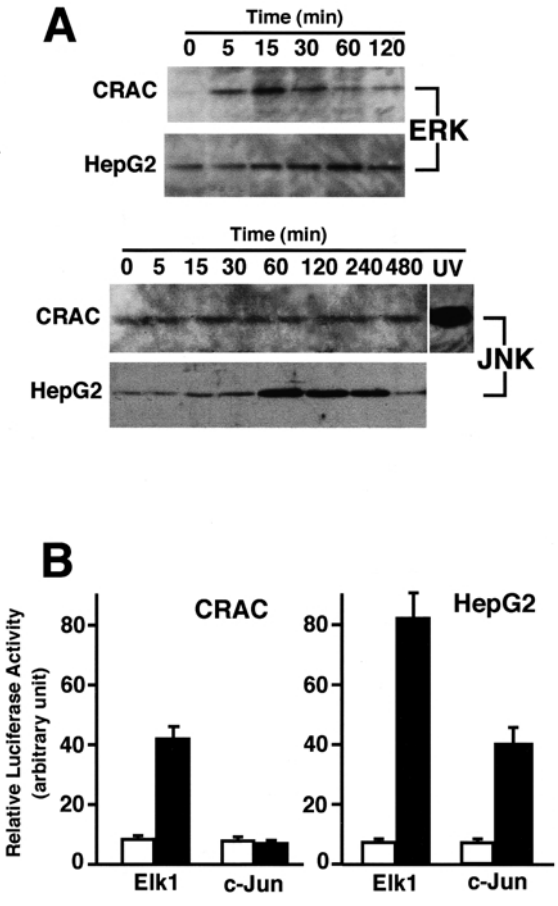

Fig. 1. TGFβ1 activates MAPK cascade in CRAC and HepG2. (A) Time-dependent activation of ERK and JNK in CRAC and HepG2 in response to TGFβ1. CRAC and HepG2 were serum-derived for 24 h and stimulated with 5 ng/ml TGFβ1 for the indicated time intervals. Equal amounts of total cell lysates were immunoprecipitated with anti-phospho-ERK antibody or c-Jun conjugated sepharose beads. *In vitro* kinase assays were performed at 30°C for 30 min using appropriate substrates as indicated. The samples were then subjected to 10% SDS-PAGE and immunoblotted with specific antibodies against phosphorylated forms of each substrate. For a positive control in JNK assay, CRAC were irradiated for 10 min with UV (25 J/m^2). (B) Trans-reporting activation of Elk1 and c-Jun by TGFβ in CRAC and HepG2. Cells were cotransfected with 100 ng/ml of pFA-Elk1 or pFA-c-Jun together with 500 ng/ml of pFR-Luc and 150 ng/ml of pCMV5-β galactosidase, cultured with (solid bars) or without (open bars) 5 ng/ml TGFβ1 for 24 h, and subsequently followed by measurement of luciferase activity. Each bar represents the mean±SEM of six samples. Luciferase activity is expressed relative to the control. Results are representative example of three independent experiments.

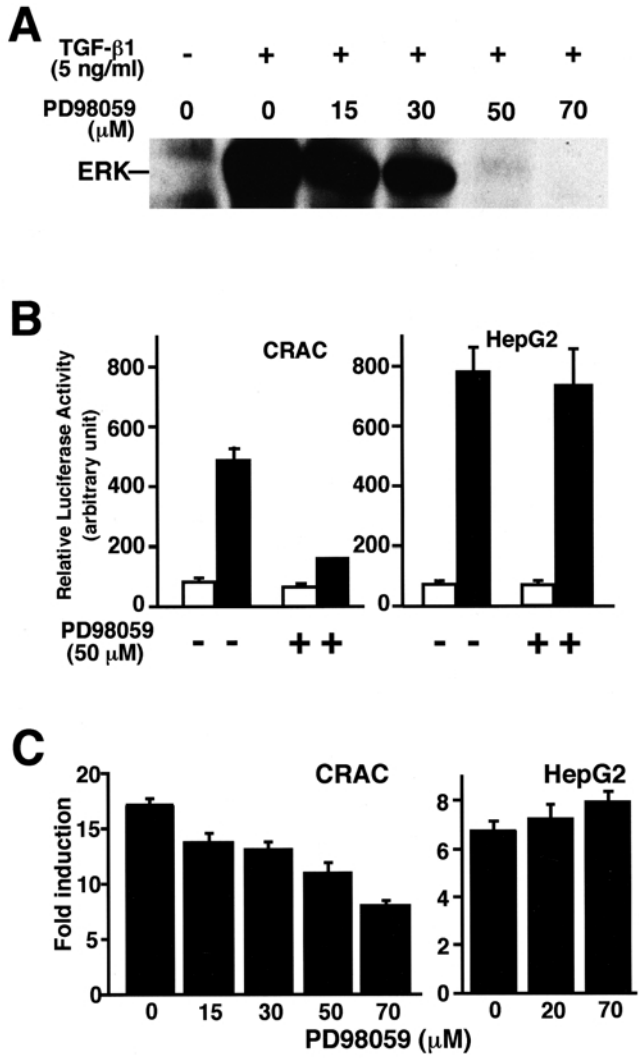

Fig. 2. Effect of specific MEK inhibitor PD98059 on TGFβ1-induced MAPK activation. (A) PD98059 inhibited TGFβ-induced ERK activation in CRAC in a dose-dependent manner. Cells were preincubated with the indicated concentrations of PD98059 for 1 h prior to stimulation by 10 ng/ml TGFβ. *In vitro* kinase assay was performed using cell lysates prepared at 15 min after stimulation. (B) PD98059 inhibited TGFβ-induced Elk1 phosphorylation of CRAC but not of HepG2. Cells transfected with pFA-Elk1, pFR-Luc and pCMV5-β galactosidase were incubated with 50 μM PD98059 together with (solid bars) or without (open bars) 5 ng/ml TGFβ for 24 h. Data are expressed as mean±SEM of relative activity measured in six samples. (C) PD98059 inhibited TGFβ1-induced 3TP transcriptional activity in CRAC but not in HepG2. Cells transfected with 500 ng/ml of p3TP-lux and 250 ng/ml of pCMV5-β galactosidase were incubated with the indicated concentrations of PD98059 together with or without 5 ng/ml TGFβ. Bars represent fold induction over the control values without TGFβ (*N*=4, mean±SEM).

lying upstream of ERK and JNK were cotransfected with 3TP-lux. In CRAC, transfection of the wild or active form of MEKK significantly inhibited TGFβ-induced 3TP activity, whereby the inhibition was dominant in active MEKK1. On the other hand, transfection of the kinase negative form of MEKK1 or stress-activated protein kinase/ERK kinase 1 (SEK1) did not change the reporter activity (Fig. 3). In HepG2 cells, overexpression of active MEKK1 resulted in a strong activation of 3TP activity in both ligand-dependent and independent induction, although wild MEKK1 did not influence the activity. Transfection of dominant forms of MEKK1 or SEK1, however, significantly diminished 3TP-lux, especially in ligand-dependent activation. These results indicate that in CRAC, ERK activation by TGFβ is independent of MEKK1 or SEK1. In contrast, HepG2 cells seem to require MEKK1 and SEK1 for TGFβ signaling.

NEGATIVE AND POSITIVE REGULATION OF 3TP-LUX ACTIVITY BY TAK1 IN CRAC AND HEPG2

Another MEKK homologue, TAK1, also participates in MAPK cascades. Therefore we studied the possible

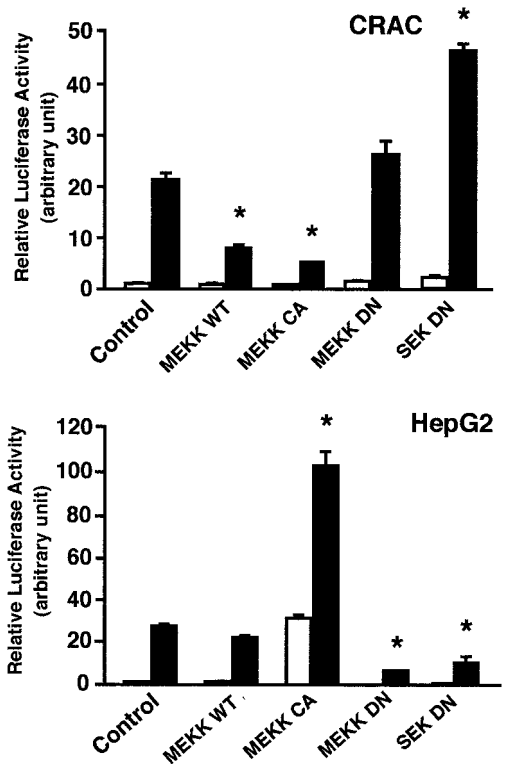

Fig. 3. MEKK and SEK1 negatively regulated TGFβ1-induced 3TP activity in CRAC, but positively regulated in HepG2. Equal amounts of the indicated expression vector or empty pCMV5 were transfected with 3TP-lux, and cells were incubated with (solid bars) or without (open bars) TGFβ (5 ng/ml). Each bar represents the mean±SEM of relative activity measured in six samples. Results are representative example of one of three independent examples. **P*<0.01; Mann–Whitney *U* test. MEKK WT; wild type MEKK, MEKK CA; constitutive active MEKK, MEKK DN; dominant negative MEKK, SEK DN; dominant negative SEK1.

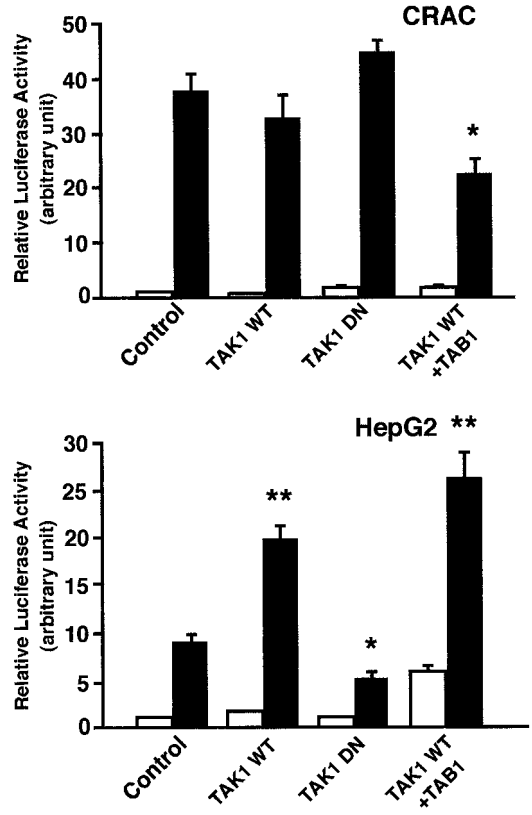

Fig. 4. TGFβ1 activated 3TP through TAK1-independent and dependent pathways in CRAC and HepG2, respectively. Equal amounts of the indicated expression vector or empty pCMV5 were transfected with 3TP-lux, and the cells were incubated with (solid bars) or without (open bars) 5 ng/ml TGFβ. Each bar represents the mean±SEM of relative activity measured in six samples. Results are representative example of one of three independent examples. **P*<0.01, ***P*<0.001; Mann–Whitney *U* test. TAK1 WT; wild type TAK1, TAK1 DN; dominant negative TAK1, TAB1; wild type TAB1.

involvement of TAK1 in TGFβ signaling in CRAC and HepG2. In CRAC, transfection of the wild or negative form of TAK1 did not significantly affect TGFβ-induced 3TP activity in CRAC (Fig. 4). In contrast, cotransfection of wild TAK1 with TAB1, an upstream activator of TAK1, resulted in strong inhibition of ligand-induced activity. In HepG2, activation of TAK1 caused a diverse effect on 3TP-lux activation compared to CRAC. Transfection of kinase-negative TAK1 decreased ligand-dependent 3TP induction, whereas wild TAK1 or a combination with TAB1 increased both the basal and fold induction. These results indicate that TAK1 also negatively and positively regulates TGFβ-induced signaling in CRAC and HepG2, respectively.

CROSS-TALK TO TGFβ-INDUCED SIGNAL TRANSDUCTION BY PKA AND PKC PATHWAYS IN CRAC

We investigated the possibility of the cross-talk to the TGFβ signaling pathway by PKA and PKC in CRAC. For this purpose, 8-bromo-cyclic AMP and forskolin, a cAMP analogue and stimulator of PKA, respectively, were used in 3TP-lux study. Incubation of CRAC with 8-bromo-cyclic AMP or forskolin decreased 3TP activity in a dose-dependent manner, although the inhibition was incomplete (Fig. 5A and B). Calphostin C, a specific inhibitor of PKC also partially decreased 3TP activity in a dose-dependent manner (Fig. 5C). To confirm that the inhibition of TGFβ signaling occurred at ERK level, we analyzed the kinase activity of ERK by *in vitro* kinase assay. Pre-treatment with calphostin C and forskolin for 1 h before TGFβ stimulation significantly decreased the kinase activity of ERK (Fig. 5D). These results indicate that PKA pathway negatively regulates TGFβ signaling by inhibiting MAPK cascade involving ERK, and that PKC activation through ERK activation is required for TGFβ signaling in CRAC.

Discussion

In this study, we demonstrated that TGFβ transmits intracellular signal molecules through two different mechanisms in CRAC and HepG2 cells. In CRAC, *in vitro* kinase assay and trans-reporting assay revealed that ERK, but not JNK, of MAPK cascade was activated by TGFβ. In addition, both 3TP luciferase induction and ERK activation were blocked by PD98059. These results indicated that TGFβ induced activation of the MEK–ERK pathway was required for TGFβ signaling in CRAC. In HepG2 cells, however, TGFβ activated JNK, but not ERK, in *in vitro* kinase assay. Although both Elk1 and c-Jun were phosphorylated by TGFβ in trans-reporting assay, the phosphorylation of Elk1 could not be blocked by PD98059, suggesting that

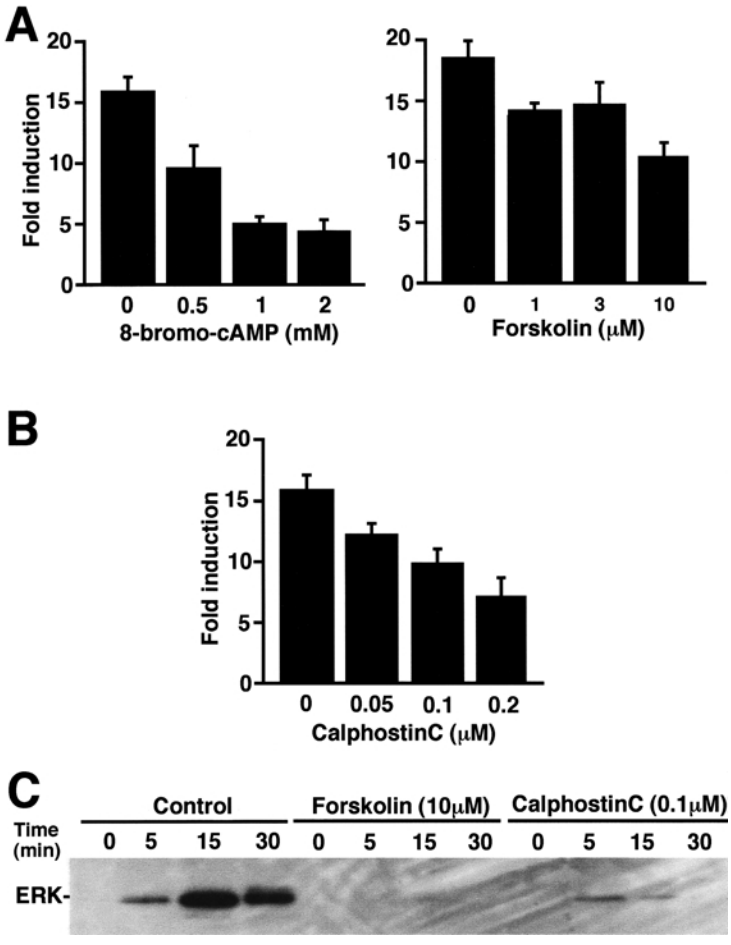

Fig. 5. Cross-talk of PKC and PKA pathways to TGFβ signaling in CRAC. (A,B) Inhibition of TGFβ-induced 3TP activity by 8-bromo-cyclic AMP or forskolin. CRAC transfected with 3TP-lux were incubated with the indicated concentrations of 8-bromo-cyclic AMP or forskolin, a cAMP analogue or an adenylate cyclase stimulator, respectively, with or without TGFβ. Bars represent fold induction by TGFβ relative to the control (N=4, mean±SEM). (C) Inhibition of TGFβ-induced 3TP activity by calphostin C. CRAC were pre-incubated in calphostin C, a specific PKC inhibitor, at the indicated concentration 1 h prior to TGFβ stimulation. Data represent fold induction relative to the control (N=4, mean±SEM). (D) Forskolin and calphostin C inhibit TGFβ-induced ERK activation. CRAC were pre-incubated in 10 μM forskolin or 0.1 μM calphostin C for 1 h prior TGFβ stimulation, and *in vitro* kinase assay was performed using cell lysates prepared at the indicated time intervals.

Elk1 phosphorylation was caused by JNK activation. Indeed, Elk1 has been confirmed to be a target of JNK as well as ERK.[18]

The response of MAPK cascades to a variety of stimuli is intricately regulated; however, these cascades could be subgrouped into three families depending on the order of activation: ERK, JNK and p38, which are the most downstream kinase, belong to MAPK. MEK, SEK and MKK belong to MAP kinase (MAPKK), while Raf1, MEKK and TAK1 belong to MAPKK kinase (MAPKKK).[19–21] Our results demonstrated that TGFβ signaling in CRAC does not depend on MEKK1, TAK1 or SEK1. Dominant negative forms of these expression vectors did not affect TGFβ-induced 3TP activation. Moreover, although overexpression of wild and active forms of these molecules activated the basal level of 3TP activity, the fold induction was blocked by these vectors, suggesting that activation of these kinases rather inhibit TGFβ signaling in CRAC. In contrast, overexpression of dominant negative forms MEKK1, TAK1 or SEK1 in HepG2 inhibited TGFβ induced 3TP-lux activation.

We first demonstrated the regulation of JNK pathway by MAPKKK in HepG2, in addition to the results of Atfi *et al*.[22] showing the involvement of JNK activation in TGFβ signaling. Considered together, our results suggest that TGFβ transmits an intracellular signal, at least in part, through the MEK–ERK pathway in CRAC, whereas activation of TAK1, MEKK1, SEK1 and JNK is required for TGFβ signaling in HepG2. Although our experiment did not identify the upstream kinases necessary for the activation of the MEK–ERK pathway in CRAC, c-Raf and/or Ras family may be involved in TGFβ signaling. However, our preliminary data suggested that overexpression of the dominant negative form of Ras (N17Ras) did not affect 3TP-lux luciferase activity in CRAC (data not shown). In addition, wortmannin phosphatidyl inositol 3-kinase inhibitor also had no effect on 3TP induction (data not shown).

TGFβ exhibits bidirectional effect on cell growth in CRAC and in HepG2, respectively. We have previously demonstrated that stimulation of CRAC with TGFβ (10 ng/ml) for 24 h increased [^{3}H] thymidine incorporation by more than seven-fold of control.[23] On the other hand, HepG2 seems

to be insensitive to, or to be suppressed by, TGFβ.[24,25] In our independent experiment, TGFβ (10 ng/ml) decreased the cell numbers of HepG2 to 60% of control, while CRAC was increased to three-fold of control (data not shown). The underlying mechanism by which TGFβ exerts the opposite effect to cell growth is so far unclear; however, differential activation of MAPK cascade depending on cell types might be a possible explanation of multipotential activity of TGFβ. Only HepG2 cells demonstrated the activation of JNK by TGFβ but not by CRAC. Indeed, it has been demonstrated in kidney distal tubule derived MDCK cells that TGFβ induces apoptosis through JNK activation.[26]

The promoter region of 3TP-lux contains three copies of TPA response element (TRE),[27] binding sites for AP-1, which is a transcriptional factor composed of c-Fos and c-Jun. Recent studies showed that Smad3 could activate TGFβ-induced transcription by binding directly with TRE.[28] In addition, Smad3 interacts with c-Fos and c-Jun cooperatively with Smad4, resulting in a strong activation of 3TP induction. A partial inhibition of 3TP-lux induction compared with ERK kinase activity, even by high concentrations of PD98059, probably means that a major part of TGFβ signaling depends on the Smad pathway. We have previously demonstrated in CRAC that transactivation of the c-fos gene by TGFβ requires a sis-inducible element (SIE), a binding site for STATs, but not a serum response element (SRE). These findings seem to contradict the requirement of MEK–ERK pathway for TGFβ signaling, However, together with our previous finding that TGFβ-induced cell growth of CRAC was inhibited by PD98059,[17] we consider that in CRAC, the MEK–ERK pathway also plays an important role in TGFβ signaling and subsequent cell growth.

Our results also demonstrated that TGFβ signaling in CRAC is regulated by PKA and PKC pathways through cross-talk mechanisms. 8-bromo-cyclic AMP and forskolin, a cAMP analogue and an adenylate cyclase activator, respectively, inhibited 3TP-lux activity and transient activation of ERK induced by TGFβ. In addition, calphostin C, a specific PKC inhibitor, also blocked 3TP-lux as well as ERK activation. These results indicate that PKC pathway is involved in TGFβ-induced 3TP-lux activation. In contrast, PKA seems to act negatively in TGFβ-activated MAPK cascade. Although the mechanism of cross-talk between PKA and TGFβ signaling has not yet been clarified, it is possible that c-Raf is a target molecule for PKA-dependent negative regulation similar to the observation identified in EGF-activated tyrosine kinase pathway.[29,30] Further experiments, including the Smad pathway and the upstream MAPK cascade leading to the activation of ERK, are required to identify the mechanisms in CRAC.

In conclusion, we have demonstrated in this study that TGFβ transduces signals through PKC-dependent MEK–ERK activation. MEKK1 inhibits TGFβ signaling; however, this inhibition does not occur through SEK1 and JNK. TAK1 is involved in TGFβ signaling. In contrast, in HepG2, activation of MEKK1, SEK1, TAK1 and JNK is required for TGFβ signaling.

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References

1. Chang SC, Hoang B, Thomas JT, Vukicevic S, Luyten FP, Ryba NJ, et al. Cartilage-derived morphogenetic proteins. New members of the transforming growth factor-β superfamily predominantly expressed in long bones during human embryonic development. J Biol Chem 1994;269:28227–34.
2. Saltis J. TGF-β: receptors and cell cycle arrest. Mol Cell Endocrinol 1996;116:227–32.
3. Moses HL, Serra R. Regulation of differentiation by TGF-β. Curr Opin Genet Dev 1996;6:581–6.
4. Solloway MJ, Robertson EJ. Early embryonic lethality in Bmp5; Bmp7 double mutant mice suggests functional redundancy within the 60A subgroup. Development 1999;126:1753–68.
5. Hoodless PA, Wrana JL. Mechanism and function of signaling by the TGFβ superfamily. Curr Top Microbiol Immunol 1998;228:235–72.
6. van Osch GJ, van den Berg WB, Hunziker EB, Hauselmann HJ. Differential effects of IGF-1 and TGF β-2 on the assembly of proteoglycans in pericellular and territorial matrix by cultured bovine articular chondrocytes. Osteoarthritis Cartilage 1998; 6:187–95.
7. Demoor FM, Redini F, Boittin M, Pujol JP. Expression of decorin and biglycan by rabbit articular chondrocytes. Effects of cytokines and phenotypic modulation. Biochim Biophys Acta 1998;1398:179–91.
8. Campbell IK, Wojta J, Novak U, Hamilton JA. Cytokine modulation of plasminogen activator inhibitor-1 (PAI-1) production by human articular cartilage and chondrocytes. Down-regulation by tumor necrosis factor α and up-regulation by transforming growth factor-β basic fibroblast growth factor. Biochim Biophys Acta 1994;1226:277–85.
9. Moldovan F, Pelletier JP, Hambor J, Cloutier JM, Martel PJ. Collagenase-3 (matrix metalloprotease 13) is preferentially localized in the deep layer of human arthritic cartilage *in situ*: *in vitro* mimicking effect by transforming growth factor β. Arthritis Rheum 1997;40:1653–61.
10. Glansbeek HL, van BH, Vitters EL, van der Kraan, et al. Stimulation of articular cartilage repair in established arthritis by local administration of transforming growth factor-β into murine knee joints. Lab Invest 1998;78:133–42.
11. Bradham DM, Horton WJ. *In vivo* cartilage formation from growth factor modulated articular chondrocytes. Clin Orthop 1998;00:239–49.
12. Tsukazaki T, Chiang TA, Davison AF, Attisano L, Wrana JL. SARA, a FYVE domain protein that recruits Smad2 to the TGFβ receptor. Cell 1998;95:779–91.
13. Visser JA, Themmen AP. Downstream factors in transforming growth factor-β family signaling. Mol Cell Endocrinol 1998;146:7–17.
14. Hocevar BA, Brown TL, Howe PH. TGFβ induces fibronectin synthesis through a c-Jun N-terminal kinase-dependent, Smad4-independent pathway. EMBO J 1999;18:1345–56.
15. Tsukazaki T, Usa T, Matsumoto T, Enomoto H, Ohtsuru A, Namba H, et al. Effect of transforming growth factor β on the insulin-like growth factor-I autocrine/paracrine axis in cultured rat articular chondrocytes. Exp Cell Res 1994;215:9–16.
16. Osaki M, Tsukazaki T, Yonekura A, Miyazaki Y, Iwasaki K, Shindo H, et al. Regulation of c-fos gene induction

and mitogenic effect of transforming growth factor β in rat articular chondrocyte. Endocr J 1999;46: 253–61.
17. Yonekura A, Osaki M, Hirota Y, Tsukazaki T, Miyazaki Y, Matsumoto T, et al. Transforming growth factor-β stimulates articular chondrocyte cell growth through p44/42 MAP kinase activation. Endocr J 1999; 46:545–53.
18. Forrer P, Tamaskovic R, Jaussi R. Enzyme-linked immunosorbent assay for measurement of JNK, ERK, and p38 kinase activities. Biol Chem 1998; 379:1101–11.
19. Minden A, Lin A, McMahon M, Lange-Carter C, Derijard B, Davis RJ, et al. Differential activation of ERK and JNK mitogen-activated protein kinases by Raf-1 and MEKK. Science 1994;266:1719–23.
20. Yan M, Dai T, Deak JC, Kyriakis JM, Zon LI, Woodgett JR, et al. Activation of stress-activated protein kinase by MEKK1 phosphorylation of its activator SEK1. Nature 1994;372:798–800.
21. Yamaguchi K, Shirakabe K, Shibuya H, Irie K, Oishi I, Ueno N, et al. Identification of a member of the MAPKKK family as a potential mediator of TGFβ signal transduction. Science 1995;270:2008–11.
22. Atfi A, Djelloul S, Chastre E, Davis R, Gespach C. Evidence for a role of Rho-like GTPases and stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) in transforming growth factor β-mediated signaling. J Biol Chem 1997;272: 1429–32.
23. Tsukazaki T, Ohtsuru A, Namba H, Oda J, Matsumoto T, Osaki M, et al. Parathyroid hormone-related protein (PTHrP) action in rat articular chondrocyte: comparison of PTH(1-34), PTHrP(1-34), PTHrP(1-141), PTHrP(100-114) and antisense oligonucleotides against PTHrP. J Endocriol 1996;150:359–68.
24. Chapekar MS, Huggett AC, Thorgeirsson SS. Growth modulatory effects of a liver-derived growth inhibitor, transforming growth factor β1, and recombinant tumor necrosis factor α, in normal and neoplastic cells. Exp Cell Res 1989;185:247–57.
25. Stolz DB, Michalopoulos GK. Synergistic enhancement of EGF, but not HGF, stimulated hepatocyte motility by TGFβ1 *in vitro*. J Cell Physiol 1997; 170:57–68.
26. Atfi A, Buisine M, Mazars A, Gespach C. Induction of apoptosis by DPC4, a transcriptional factor regulated by transforming growth factor β through stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) signaling pathway. J Biol Chem 1997; 272:24731–34.
27. Wrana JL, Attisano L, Carcamo J, Zentella A, Doody J, Laiho M, et al. TGFβ signals through a heteromeric protein kinase receptor complex. Cell 1992;71: 1003–14.
28. Zhang Y, Feng XH, Derynck R. Smad3 and Smad4 cooperate with c-Jun/c-Fos to mediate TGF-β-induced transcription. Nature 1998;394:909–13.
29. Cook SJ, McCormick F. Inhibition by cAMP of Ras-dependent activation of Raf. Science 1993;262: 1069–72.
30. Wu J, Dent P, Jelinek T, Wolfman A, Weber MJ, Sturgill TW. Inhibition of the EGF-activated MAP kinase signaling pathway by adenosine 3′,5′-monophosphate. Science 1993;262:1065–9.

Appendix

ABBREVIATIONS

BMP: bone morphogenic protein
CDGF: cartilage-derived growth factor
CRAC: cultured rat articular chondrocytes
ERK: extracellular regulated kinase
JNK: c-Jun N-terminal kinase
MAPK: mitogen-activated protein kinase
MAPKKK: MAPK kinase kinase
MEK: MAPK/ERK kinase
MEKK1: MEK kinase 1
TGFβ: transforming growth factor β